CONTRACT NO: DAMD17-91-C-1096

TITLE: VASOPRESSIN RECEPTOR SIGNALING AND CYCLING OF WATER CHANNELS IN RENAL EPITHELIA (HBC)

SUBTITLE: Retrieval of Water Channels in Toad Urinary Bladders Following Withdrawal of Vasopressin and Mezerein

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REPORT DATE: August 27, 1993

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Development Command, Fort Detrick
Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	27 August 1993	Annual Report (8/1/92 - 7/31/93)

4. TITLE AND SUBTITLE
Vasopressin Receptor Signaling and Cycling of Water Channels in Renal Epithelia (HBC)

5. FUNDING NUMBERS
Contract No.
DAMD17-91-C-1096

61102A
3M161102B515.CA
WUDA335934

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research & Development Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Subtitle: Retrieval of Water Channels in Toad Urinary Bladders Following Withdrawal of Vasopressin and Mezerein

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The role of renal cortical epithelia in fluid reabsorption and regulation of urinary volume is crucial in maintaining homeostasis with the hormone vasopressin (ADH) playing a critical role. We have been studying the mechanisms of transmembrane water flow by exocytosis and retrieval of water channels by endocytosis as induced by vasopressin. Water flow processes are also induced by mezerein (MZ), an activator of protein kinase C (PKC), in a manner very similar to what were produced by ADH. Scanning and transmission electron microscopy demonstrated that the membrane recovery process was associated with an induction of invaginations only at the apical plasma membranes involving many granular epithelial cells. During endocytosis, apical membranes underwent transition with a loss of both microridges and microvilli prior to membrane restoration to the unstimulated

14. SUBJECT TERMS
Water flow; ADA (vasopressin); Protein Kinase C; Endocytosis; Water Channels; Channel retrieval, RAD III

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Block 13. Abstract (continued)

state. Time-course studies indicated that increases in cell-surface endocytosis were correlated with a decrease in water loss from the urinary bladder sacs. These observations suggested that the apical membrane remodeling was crucial for the restoration of membrane permeability following ADH or MZ-activation and termination, and further suggest that PKC may participate in the overall scheme of the cycling of water channels.

FOREWORD

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8/27/93

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RETRIEVAL OF WATER CHANNELS IN TOAD URINARY BLADDERS FOLLOWING WITHDRAWAL OF VASOPRESSIN AND MEZEREIN

Abstract: Antidiuretic Hormone (ADH, vasopressin) increases transepithelial water flow in renal epithelia by a process that involves the insertion of water channels into the apical membrane. Presently, changes in surface structure in a ADH responsive tissue, the toad urinary bladder, were examined using the techniques of scanning electron microscopy under conditions that promote endocytosis of water channels during recovery from a hormonal challenge. Hemibladders were set up as sacs with an imposed osmotic gradient. The control sacs received no hormone treatment, whereas the other sacs were stimulated with 100 mU/ml ADH or Mezerein (MZ 10^{-6}M) for 10 or 15 min to induce exocytosis and enhanced water flow. ADH or MZ was then washed from the tissues with fresh buffer rinses to abolish the hormone or MZ actions. These tissues were then allowed to recover for 5, 10, 15, 20, 30 and 60 min. During these time periods, water channels gradually recovered intracellularly by a process of endocytosis. This time period was called the retrieval period. At specified time intervals, tissues were fixed and processed for SEM or embedded in epon for ultrathin sectioning for TEM studies. Control tissues, regardless of the length of time, showed little or no sign of surface remodeling that was indicative of endocytosis during pre or post-buffer washes, whereas the ADH and MZ-treated tissues showed a time-dependent remodeling of the apical membrane during activation and following removal of the hormone and MZ during the retrieval period. At the 10 min retrieval period following withdrawal of ADH, greater than 47% of the granular cells showed

extensive surface remodeling compared to 7.78% cells in MZ-exposed tissues. By 30 and 60 min post hormone treatment during recovery, fewer than 23% of granular cells showed signs of surface membrane changes, indicating that the tissue was returning the apical membrane surface to the unstimulated state. During endocytosis, apical membranes undergo transition, with a loss of both microridges and microvilli prior to membrane restoration. These observations suggest that apical membrane remodeling is crucial for the restoration of membrane permeability following ADH or MZ-activation and termination, and further suggests that protein kinase C (PKC) may participate in the overall process of cycling of water channels (aggrephores) in toad urinary bladders.

Introduction

Eucaryotic cells, through the process of specialization, have acquired complex, yet integrated molecular and cellular machineries to recycle plasma membrane components and resident proteins, as a means to regulate a variety of cellular functions. Internal membrane shuttling in the amphibian urinary bladder, as well as in renal cortical cells, for the regulation of transepithelial water flow includes two important events: exo- and endocytosis (Fig.1). The exocytotic process involves selective packaging of the water channel containing vesicles for export toward the apical membrane where they fuse following stimulation of bladder tissues with antidiuretic hormone (ADH, Vasopressin). The process of endocytosis is involved in ferrying the membranes as clathrin coated vesicles in the reverse direction, i.e. toward the cell interior following withdrawal of hormone (Schmid, 1992). In our laboratories,

we have conducted experimental studies on transmembrane osmotic water flow using toad urinary bladders, an ADH responsive tissue. A variety of electron microscopic techniques were used to gain insight into the underlying mechanism involved in the cellular processes of exo- and endocytosis as related to water transport.

ADH through V_2 and V_1 receptors enhances the transmembrane osmotic water flow across the apical membrane of renal epithelia. ADH V_2 receptor transduction results in the stimulation of adenylate cyclase, an increase in cAMP and the activation of protein Kinase A (PKA) (Handler and Orloff 1973, 1981; Schlondorff and Levine, 1985). These biochemical events are believed to induce shifts in the cellular ultrastructure that results in mucosal membrane reorganization to numerous microvilli from the normal phase of microridges (DiBona, 1981; Mia *et al.* 1983, 1988a,b; Mills and Malick, 1978; Spinelli *et al.*, 1975). Propagation of microvilli occurs simultaneously with apical membrane fusion of proteinacious water channels. These water channels appear as specific aggregates of intramembrane particles arranged in stellar arrays when viewed by freeze-fracture (Chevalier *et al.*, 1974; Hays *et al.*, 1985; Kachadorian *et al.*, 1978; Mia *et al.*, 1991b; Wade *et al.*, 1981). Amphibian epithelia also contain a vasopressin V_1 receptor coupled to phosphoinositide metabolism with the release of inositol trisphosphate, a mobilizer of intracellular calcium ions, and diacylglycerol, an activator of protein kinase C (PKC) (Yorio and Satumtira, 1989). Data from our laboratories suggests that the vasopressin V_1 receptor cascade may play an integral role in the insertion of water channels into the apical membrane, likely through activation of PKC. Scanning electron microscopic

studies demonstrated the induction of microvilli at the apical membrane of the toad urinary bladders by mezerein, a non-phorbol activator of PKC (Mia *et al.*, 1989). These observations coupled with freeze fracture studies and immunogold labeling of PKC isozymes (Mia *et al.* 1991b, 1992), suggested that both ADH V_2 and V_1 receptors may participate in the overall scheme of increased osmotic water flow stimulated by hormone. Following the termination of the action of ADH, the granular cells of toad urinary bladders undergo membrane internalization through the transport of endocytic vesicles (see Brown, 1989; Coleman *et al.*, 1987; Schmid, 1992). Little is known about the mechanism of apical membrane remodeling that occurs during endocytosis (Mia *et al.* 1993a,b,c). Recently, we demonstrated that the membrane internaliation process, through the transport of endocytic vesicles, can be increased by actions following removal of mezerein (MZ) in a manner very similar to that produced by ADH. The present study uses techniques of scanning electron microscopy (SEM) combined with transmission electron microscopy (TEM) as a means to determine the surface and intracellular mechanisms associated with the regulation of membrane recycling in amphibian urinary bladders, a renal model epithelia, during endocytotic retrieval following removal of hormone, ADH or MZ. This report is part of our continuous effort in probing the overall mechanisms of the regulation of water flow by ADH.

Methods

Tropical toads, Bufo marinus, were purchased from NASCO, Ft. Atkinson, WI or from Carolina Biological Supply House, Burlington, N.C., and were maintained at

28°C, fed live crickets biweekly and had free access to water.

Experimental Protocol:

Whole Bladder Tissue

Urinary hemibladders, surgically excised from doubly-pithed toads, were set up as sacs at the ends of glass tubes such as in Figure 2, and were placed in aerated isotonic normal Ringer's solution as previously reported (Mia *et al.* 1983, 1991a). The composition of the Ringer's solution was as follows (in millimoles per liter): NaCl, 111.0; KCl, 3.35; CaCl, 2.7: $MgCl_2$, 0.5; $NaHCO_3$, 4.0 and glucose, 5.0. Hemibladders were allowed to equilibrate for 30 min in isotonic Ringer's solution, and at time of experimental intervention, an osmotic gradient was instituted using 1/10 dilution of Ringer's solution in the mucosal cavity. Control tissues were maintained in Ringer's solution, whereas experimental tissues received 100 mU/ml (ADH) or MZ 10^{-6}M for 10 min. Following withdrawal of ADH or MZ after 10 min stimulation, by two quick buffer washes, tissues were allowed to incubate for 5,10 and 20 min without the hormone or mezerein allowing the apical membrane retrieval by endocytosis. A similar experiment was also conducted exposing toad urinary bladder sacs to 100 mU/ml ADH for 15 min, and then retrieved for 15, 30 and 60 min following withdrawal of vasopressin. During this retrieval period water loss from the sac was also measured using a gravimetric procedure (Bentley, 1958). At the end of the experimental procedure, hemibladder sacs were cut and immediately placed into 2% glutaraldehyde fixative in PIPES buffer (0.02M) in individual glass vials. The fixative was then replaced with fresh glutaraldehyde and fixation was allowed to

continue for 1 hr. Post-fixation was carried out using 1% osmium tetroxide for 1 hr. Fixed tissues, washed in PIPES buffer, were processed for SEM and TEM. For SEM preparations, tissues were placed in glass vials and processed through exchanges of graded acetone and liquefied Peldri II for critical point drying. Dehydrated tissues were then submerged into fresh liquefied Peldri II and allowed to stand for 1-2 hr for complete removal of acetone and impregnation of Peldri II for critical point drying. Liquid Peldri II was allowed to solidify in closed vials by the gradual dropping of temperature below 23°C (75°F). Once the Peldri II solidified, the vial caps were removed to allow slow sublimation either in a freeze drier or in a fume hood (Mia *et al.*, 1993a.b.c). Each dried tissue sample was mounted on an aluminum stub using silver paint and was surface coated with gold in a sputter coater in an argon environment for SEM observations. For TEM, fixed tissues were minced and dehydrated using exchanges of ethanol and propylene oxide prior to embedding in Luft's epon (Mia *et al.* 1987, 1991a). Ultrathin sections were prepared with a diamond knife using an ultramicrotome. These sections were placed on bare copper or nickel grids, stained with uranyl acetate and lead citrate and viewed in the transmission electron microscope.

Determination of Surface Changes

For morphometric quantitation, between four and six pictures for each treatment were taken at 1500X and 4000X magnifications using SEM. The total number of normal and treated cells in each picture image was counted and averaged

to show results in percentages. The statistical analysis of each treatment group (control versus ADH) was made on a Texas Instruments Professional Computer using the Trajectories statistical program (DBI Software, Mount Pleasant,MI) as presented in Figure 3. The ANOVA and Fischer's PLSD (Protected Least Significant Difference) were performed with Stateview (Abacus Concepts, Inc., Berkeley, CA) on an Apple MacIntosh IIsi microcomputer. Descriptive statistical data were calculated using between 4 and 6 images, and the results presented are as mean $\pm$ 1 standard deviation. The results of the ANOVA and Fischer's PLSD showed that there was no significant difference among the controls, whereas a significant difference at the 5% level was seen between the controls and the ADH-treated tissues. Figure 3 shows the percent of ADH and MZ-induced endocytosed cells against the control tissues.

Drugs

8-Arginine vasopressin was purchased from Sigma Chemical Co., St. Louis, MO. The concentration of ADH used in these experiments was 100 mU/ml, and was added to the serosal side of the hemibladder sacs and the concentration of MZ was 10^{-6}M and was added to the urinary cavity. Control tissues were retained in Ringer's solution for comparative analysis.

Results

The technique of scanning electron microscopy was used to evaluate the surface substructure changes that occur following removal of hormone and mezerein subsequent to enhanced water flow in toad urinary bladder tissues. The main purpose

of this series of experimental studies was to probe into the mechanism of membrane internalization following withdrawal of vasopressin and mezerein through a series of fresh buffer rinses, and to evaluate the role of protein kinase C in apical membrane retrieval through the process of endocytosis. Figures 4 and 5 depict the microstructure environment of the apical membrane of the control tissues (no hormone, Fig. 4) and ADH-exposed tissues (less than 1 min treatment, Fig. 5) in the presence of an osmotic gradient. At this time period, the control, as well as the ADH-exposed tissues, as expected, showed little or no visible changes in the apical surface microstructure, similar to what has been reported previously (Mia *et al.* 1983, 1987). Therefore, a vast majority of the cells had a mucosal plasma membrane of primarily a microridge structure. The granular cells, both in the control and ADH-stimulated tissues, showed little or no visible tissue swelling. However, the goblet cells, facing the urinary lumen, appeared to have puffed out, likely due to exposure of the tissues to the osmotic gradient. Granular cells, under an identical osmotic gradient, responded to challenges with 100 mU/ml ADH for 10 min with pronounced tissue swelling and the propagation of numerous microvilli over the entire apical membrane surface (Fig. 6). Similarly, mezerein treatment induced microvilli formation very similar to what was produced by ADH stimulation (Fig. 7). Control tissues (no ADH or MZ), during this same time period and under an imposed osmotic gradient, tend to retain their normal unstimulated surface structure of predominantly microridges. These cells show little tissue swelling consistent with previous observations (Mia *et al.* 1983, 1987). Control tissues, retained in Ringer's solution for 10 min, also showed little or no

evidence of apical membrane invaginations, whereas ADH stimulated tissues at 10 min, with no buffer washes, showed 15 percent of the cells with evidence of apical surface invaginations (Fig. 3). Such membrane surface invaginations (Fig. 8), associated with continuous ADH-stimulation, may have been the result of down-regulation of the membrane permeability response. Such a down-regulation of membrane permeability was not usually evident at 10 min stimulation of tissues treated with MZ.

In another series of experiments, both control and ADH-challenged tissues, following incubation for 15 min, received two quick fresh buffer rinses to abolish the hormone actions from the ADH-challenged tissues. These tissues were then allowed to retrieve for 15, 30 and 60 min. The SEM analysis of the apical surface morphology revealed that the control tissues, regardless of the length of time under an osmotic gradient showed little or no sign of apical membrane manifestations following buffer reinses during the retrieval period (Fig. 9). Many scanning electron micrographs of control tissues, representing each stage of 15, 30 and 60 min retrieval periods, were studied and found to show a general lack of apical membrane surface changes indicative of endocytosis (see Fig. 9). In contrast, comparable tissues, following removal of ADH or MZ resulted in apical membrane invaginations consistant with the retrieval of water channels by endocytosis. The molecular and cellular mechanisms that may interplay to induce this apical membrane remodeling is unknown. Figures 10 and 11 depict the surface configuration, as displayed by ADH and MZ-stimulated tissues, during a 5 min retrieval period. A number of granular cells developed

prominent invaginations over the mucosal surface area. The percent of the granular cells showing the loss of surface villi as an index of endocytosis was 3.27% in control as opposed to 31.28% and 4.19% in the ADH and MZ-stimulated tissues respectively (Fig. 3).

The apical membrane retrieval process in toad bladder tissues, as triggered by removal of hormone ADH or MZ, appeared to be progressive. Therefore, various stages of endocytosis during each retrieval period may be encountered in cells over a single tissue surface. The retrieval process begins with several slight surface invaginations each of which exhibits elevated margins (Figs. 10, 11). These invaginations, in most cells, were found to coalesce laterally to form a single large invagination across the entire membrane surface of the granular cells. Ultrathin sections, through such membrane invaginations, when examined in the transmission electron microscope, demonstrated the presence of low surface invaginations (Fig. 12), analogous to that seen in SEM (Fig. 10). At 10, 15 or 20 min retrieval periods, following withdrawal of ADH or MZ, the toad urinary bladder surfaces were seen to have a large number of endocytotic pits (Figs. 13,14). Although endocytosed pits were induced in MZ-treated tissues during post buffer washes, their appearance was slower compared to ADH-exposed tissues. Therefore, at 10 min retrieval, fewer cells in MZ-treated tissues showed surface invaginations (Fig.15) in contrast to a greater number of ADH-treated cells (Fig. 13). Cell counts at 10 min retrieval, indicated that fewer than 8% of the cells showed surface invaginations and putative endocytosis in MZ-exposed tissues (n = 6), as compared to 45% of the cells in ADH-stimulated

tissues (n = 6). The incidence of proposed endocytosis in MZ and ADH-treated tissues reverses with time of retrieval. Therefore, at 20 min retrieval, 35% of the MZ cells showed endocytosis (Fig. 16) compared to 17% of the ADH-treated cells. Many endocytosed pits, which appear to be internalized into the cytoplasm, are large and can often be seen in the ultrathin sections of TEM (Fig. 17, arrows). These endocytotic pits are only found along the apical membranes and not associated with the basolateral or basal plasma membrane. However, coated vesicles, originating from the basal as well as the apical membrane, were seen to be present in the cytosol of some ADH-stimulated granular cells (Fig. 18, arrows). Due to the enlargement of endocytosed vacuoles, the nuclei in some cells may be pressed against the basal membrane, and, as a result, the nuclei may assume a variety of shapes surrounded by endocytotic vacuoles (Fig. 17).

Control tissues, under an imposed osmotic gradient for 15 min, had an average fluid loss from the bladder sac of 2.1 ul/min (n = 4), whereas ADH-challenged tissues had 41 ul/min fluid loss (n = 4). Apical membrane surface changes in ADH-stimulated tissues reached a peak at 10 or 15 min into the retrieval period, with a percent of cells involved at 45.9% and 63.7%, respectively. Whereas in the control tissues, less than 1% of the cells were involved (Fig. 3). During the process of the formation of invaginations, apical membrane surfaces tend to undergo membrane remodeling with the loss of both microvilli and microridges in both ADH and MZ-stimulated tissues (Figs. 13,14,16, arrows) leaving flattened membrane surfaces with no visible microstructure. In ultrathin sections, such membranes appear to contain no micro-

ridges or microvilli (Fig. 17). This phase of membrane disposition may represent a membrane transition during transformation of microvilli into microridges.

The recovery of the water channels from apical membranes in ADH or MZ-exposed tissues begins soon after the withdrawal of ADH or MZ. Such membrane remodeling, however, was gradual, as not all the cells showed full restoration of the apical membrane even after 20, 30 or 60 min following withdrawal of ADH. Global surface views of apical membranes at 20, 30 and 60 min retrieval periods following ADH or MZ removal show that, although a large portion of the granular cells have restored surface morphology as in the pre-hormone normal state, a few cells still show the presence of surface remodeling (Fig. 19). A closer view is presented in Figure 20 to demonstrate the membrane restoration in granular cells showing the reorganization pattern of the microridges surrounding the endocytosed pits. During the 20 min retrieval period, the number of individual cells showing endocytosis in MZ-stimulated tissues was found to be 17% and 35% respectively. During 30 and 60 min retrieval periods, the number of the involved cells in ADH- stimulated tissues was found to be 27% and 23.12% respectively versus fewer than 2% cells in the control tissues (Fig. 3).

Discussion

Several observations have prompted our attention to undertake this current series of experiments on the phenomenon of membrane retrieval and membrane remodeling in toad urinary bladder tissues. The cellular events leading to the process of endocytosis have been recently investigated and the biochemical characterization

of the clathrin coated pits associated with endocytosis in a variety of tissues has also been described (Brown, 1989; Pearse and Crowther, 1987; Schmid, 1992). However, there has been little information on examining the process of endocytosis using the techniques of SEM to unveil the external membrane remodeling that may be associated with the endocytotic retrieval of membrane components, i.e. water channels, following removal of vasopressin (Brown, 1989, Brown *et al.,* 1990, Coleman *et al.,* 1987). In addition, it was not known if PKC was involved in the retrieval processduring endocytosis. The current observations suggest that the apical plasma membrane surface of toad urinary bladders, we found that the apical plasma membrane passes through stages of membrane remodeling that is coincident with the retrieval of water channels prior to the restoration of the membrane to its normal phase containing microridges. In addition, the processes of exo- and endocytosis, with reference to surface fine structures, have not been comparatively evaluated in time-course studies using a single tissue system. This is particularly relevant to these two processes involved in the overall intracellular cycling of water channels in ADH responsive tissue. Hence, the current research reports on both the processes of exo- and endocytosis using the toad urinary bladder tissues as an ADH responsive model membrane.

The plasma membrane cycling of water channels in amphibian urinary bladder tissues, as well as in the renal cortical cells, stimulated by ADH, are accomplished by cellular events involving exo- and endocytosis. The process of exocytosis has received adequate attention and appears to involve the export of proteinacious water

channels or aggrephores, normally found in the cytosol of the unstimulated bladder tissues to the apical plasma membrane for incorporation as a means to enhance water flow upon stimulation by ADH (Humbert *et al.,* 1977; Muller and kachadorian, 1984; Wade, 1978). Enhanced apical membrane water permeability by ADH is correlated with a restructuring of the apical membrane microridges into numerous microvilli with an increase in membrane surface area (DiBona, 1981; DiBona *et al.,* 1969; Hays, 1983; Spinelli *et al.* 1975; Mia *et al.* 1983, 1987, 1988a). These cellular ultrastructural events are thought to occur through ADH V_2 receptors coupled to the activation of adenylate cyclase, an increase in cAMP and the activation of protein Kinase A (Handler and Orloff 1973, 1981; Schlondorff and Levine, 1985). Amphibian epithelia also contain an ADH V_1 receptor coupled to phosphoinositide metabolism and inositol triphosphate release (Yorio *et al.,* 1985; Yorio and Satumtira, 1989), which appears to play a functional role in the overall scheme of transepithelial aqueous flow possibly through the activation of PKC. It has been shown that mezerein (MZ), a non-phorbol activator of PKC, increased transepithelial water flow when added to the mucosal surface. Although the magnitude of water flow was less, and occurred over a longer period as compared to ADH-stimulated tissues, the response was reminiscent to that seen with hormone (Yorio and Satumtira, 1989). A series of ultrastructural experiments also revealed that this enhanced water flow induced by mezerein was indeed correlated with the propagation of apical microvilli and the change in the cellular distribution of PKC isozymes as determined by immunogold localization techniques (Mia *et al.* 1991b, 1992). It has been proposed by our laboratory that part

of the initiation of transepithelial water flow by ADH involves V_1 receptors and the activation of PKC. The water channels (aggrephores) are likely transported from the cytosal to the apical membrane domain through an involvement of PKC. Our observations involving the role of PKC during enhanced transepithelial water transport correlated with cytomorphological changes and concurrent apical membrane surface remodeling, suggesting that PKC may play an integral role in the phenomenon of membrane recycling.

The process of endocytosis in amphibian bladder tissues has been described (Muller and Kachadorian, 1984; Ding *et al.,* 1985; Harris *et al.,* 1986; Coleman *et al.,* 1987). Following stimulation of tissues by hormone, the membrane permeability to water declines with time and the tissue looses its responsiveness to ADH. During this period, membrane recycling takes place resulting in a membrane remodeling and a return of the apical membrane to the normal unstimulated state. During this membrane restoration process it is assumed water channels are retrieved by endocytosis. It has been predicted that the exocytotic membranes with intact particle aggregates may be retrieved during endocytosis. Several studies on endocytosis, using amphibian urinary bladders, have shown evidence of membrane retrieval of intact particle aggregates using specific protein markers as identification of aggregate proteins (Harris *et al.,* 1986). Additional evidence for retrieval of water channels had come from ultrathin section and freeze fracture techniques using selectively labeled colloidal gold and horseradish peroxidase as tracers to localize membrane particle aggregates (Coleman *et al.,* 1987). While these studies involve the cellular

localization of retrieved aggrephores following withdrawal of ADH, there are no studies on surface changes during this process nor time-course studies reported. Our present time-course studies provide insight into the membrane surface behavior following withdrawal of ADH. During this process, granular epithelial cell membranes tend to invaginate toward the cellular interior as an initial response to ADH withdrawal. This could result from the loss of membrane permeability to water, or as an initial cytosolic signal to begin the retrieval process. Since transepithelial water flow is maintained during the period, it is highly unlikely that it is due to changes in membrane permeability. The membrane internalization process appears to enter into transitional stages resulting in the loss of the surface microstructures (microvilli) prior to the restoration of the apical membrane to one predominantly containing microridges. The restoration of apical membranes is correlated with an inhibition of transepithelial water transport as expected across the plasma membrane. It is also noteworthy that even when hormone is continually present there appears to be a down-regulation of membrane permeability and an internalization of membrane water channels. Therefore, additional unknown mechanisms may act to bring about a return of the membrane to its normal state. The present observations also indicate and reinforce our previous observations that protein kinase C may be involved in the insertion of membrane water channenls during exocytosis, as similar endocytotic processing was seen following withdrawal of mezerein as occurred with ADH.

Additionally, we are in search of a membrane model preferably renal cortical cells, grown in tissue culture, for utilization in studies of transepithelial water flow

involving both exo- and endocytosis. Such in vitro models will provide for biochemical as well as cytological studies, for a more accurate evaluation of the water transport process under controlled experimental conditions.

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Key to Figures

Fig. 1. Schematic representation of unstimulated, ADH or MZ-stimulated and retrieval cells showing possible biochemical and cellular activities involving the cycling of water channels in toad urinary bladders.

Fig. 2. Schematic representation of toad urinary bladder set-up as sac for experiments for studying water transport process.

Fig 3. Data showing the percent of the endocytosed cells in ADH and MZ-stimulated tissues versus control for 10 or 15 min with retrieval periods of 5, 10, 20, 30 and 60 min.

Fig. 4. Control toad urinary bladder with imposed osmotic gradient showing predominant microridges. X3,000.

Fig. 5. ADH-stimulated toad bladder tissue fixed immediately upon exposure to ADH and osmotic gradient showing tissue swelling and puffing of the goblet cells. X2,000.

Fig. 6. Toad urinary bladder stimulated with 100 mU/ml ADH for 10 min showing propagation of numerous microvilli. X5,300.

Fig. 7. Toad urinary bladder stimulated with MZ 10^{-6}M for 10 min showing numerous microvilli. X3,000.

Fig. 8. Toad urinary bladder under osmotic gradient stimulated for 15 min showing membrane invagination due to down regulation of membrane permeability. X3,000.

Fig. 9. Control toad urinary bladder under osmotic gradient for 15 min and then retrieved for 30 min showing no membrane invagination. X3,700.

Fig. 10. Toad urinary bladder stimulated with 100mU/ml ADH for 10 min and retrieved for 5 min showing initiation of membrane internalization for 10 min and retrieved for 5 min. X5,360.

Fig. 11. MZ-stimulated toad urinary bladder showing apical membrane invagination following withdrawal of MZ. X3,000.

Fig. 12. Ultrathin section of toad urinary bladder sac stimulated for 10 min with ADH and retrieved for 5 min showing the membrane internalization by endocytosis. X13,300.

Fig. 13. Toad urinary bladder stimulated for 10 min with ADH and retrieved for 10 min following withdrawal of ADH showing membrane internalization by endocytosis. X2,000.

Fig. 14. Toad urinary bladder stimulated for 10 min with ADH and retrieved for 20

min showing various stages of endocytosis and membrane restoration. X930.

Fig. 15. Toad urinary bladder stimulated by MZ for 10 min and retrieved for 10 min showing endocytosed cells. X3,000.

Fig. 16. Toad urinary bladder sac stimulated by MZ for 10 min and retrieved for 20 min showing endocytosis and restoration of membranes. X3,000.

Fig. 17. Toad urinary bladder stimulated for 10 min with ADH and retrieved for 20 min showing large internalized vacuoles and change in the nuclear shape in ultrathin sections. X6,650.

Fig. 18. Toad urinary bladder stimulated with ADH for 10 min and retrieved for 20 min showing coated vesicles (arrow) and budding of vesicle from the basal plasma membrane. X40,000.

Fig. 19. Tissue stimulated with ADH for 15 min and retrieved for 30 min showing recovery of membrane with a few endocytosed cells. X2,000.

Fig. 20. Toad urinary bladder treated with MZ for 10 min and retrieved for 20 min showing details of membrane restoration. X8,000.

OTHER MILESTONES

Under this research project on biomedicine, three African-American undergraduate students, Chasity Robinson, Kristi Henderson and Sharla Hood received training in cellular and endocrine biology, tissue culture, fluorescent and electron microscopy. During this summer, 1993, Chasity Robinson received training in water

transport studies under Dr. Thomas Yorio, Kristi Henderson in the refinement of the techniques of gel electrophoresis under Dr. James Dzandu, and Sharla Hood in the Health-Career Opportunity Program (HCOP), all at the University of North Texas Health Science Center at Fort Worth (TCOM), Texas. Three new students have undertaken research and career enrichment under Dr. A.J. Mia at Jarvis Christian College, and Amanda Davidson, Research Technician continues to assist in research and training students in the research laboratories. In addition, students receive training in computer use and data plotting, and photo printing techniques using light and electron micrographs. Students are also given independent research projects for publishable research contributions. Pamler Thompson, an African-American, received graduate training at the University of North Texas Health Science Center in Ft. Worth working under the mentorship of Dr. Thomas Yorio, the Co-PI.

Research Contributions:

Full-length paper accepted:

Candia, O., Mia, A.J. and Yorio, T. 1993. Influence of filter supports on transport characteristics of cultured A6 Kidney cells. Am. J. Physiol. (in press).

Full-length paper submitted:

Mia, A.J., Oakford, L.X. and Yorio, T. 1993. Surface membrane remodeling following removal of vasopressin in toad urinary bladders. Tissue and Cell.

Full-length paper in preparation:

Role of protein Kinase C in recycling of water channels in toad urinary bladders.

Published Abstracts:

a) Mia, A.J., Oakford, L.X. and Yorio, T., 1992. Surface substructural changes of renal A6 cells on filter supports following activation of PKC. Mol. Biol. Cell. 3, 228a.

b) Mia, A.J., Oakford, L.X., Finkley, A.E., Davidson, A.D. and Yorio, T. 1993. SEM studies of membrane recycling in vasopressin-stimulated toad urinary bladders. Proc. Soc. Exptl. Biol. Med. 203, 258.

c) Mia, A.J., Oakford, L.X., Yancy, H.F., Davidson, A.D. and Yorio, T. 1993. Evidence of endocytosis in toad urinary bladders as revealed by SEM. FASEB J. 7, A577.

d) Mia, A.J., Oakford, L.X., Hayes, S.C., Davidson, A.D. and Yorio, T. 1993. Membrane dynamics during endocytosis in toad urinary bladders as visualized by SEM. Scanning, 15, Suppl III, 110-111.

e) Mia, A.J., Davidson, A.D., Oakford, L.X. and Yorio, T. 1993. SEM studies of membrane endocytosis in toad urinary bladders following withdrawal of vasopressin. Microscopic Soc. Am. 474-475.

f) Mia, A.J., Gunter, S., Johnson, S., Franklin, J., Tuck, T., Gardner, D. and Yorio, T. 1993. Immunofluorescent detection of PKC alpha in A6 amphibian kidney cells. Submitted: Am. Soc. Cell Biol.

g) Mia, A.J., Robinson, C., Bolden, A., Davidson, A.D., Oakford, L.X. and Yorio, T. 1993. Membrane remodeling by endocytosis in toad urinary bladders under sustained osmotic gradient. Submitted: Am. Soc. Cell Biol.

Research in Progress:

a) Immunofluorescent detection of microfilaments in A6 cultured amphibian kidney cells.

b) Possible induction of endocytosis in A6 cultured amphibian kidney cells.

c) Immunofluorescent detection of Protein Kinase C beta and gamma isozymes in A6 cultured amphibian kidney cells.

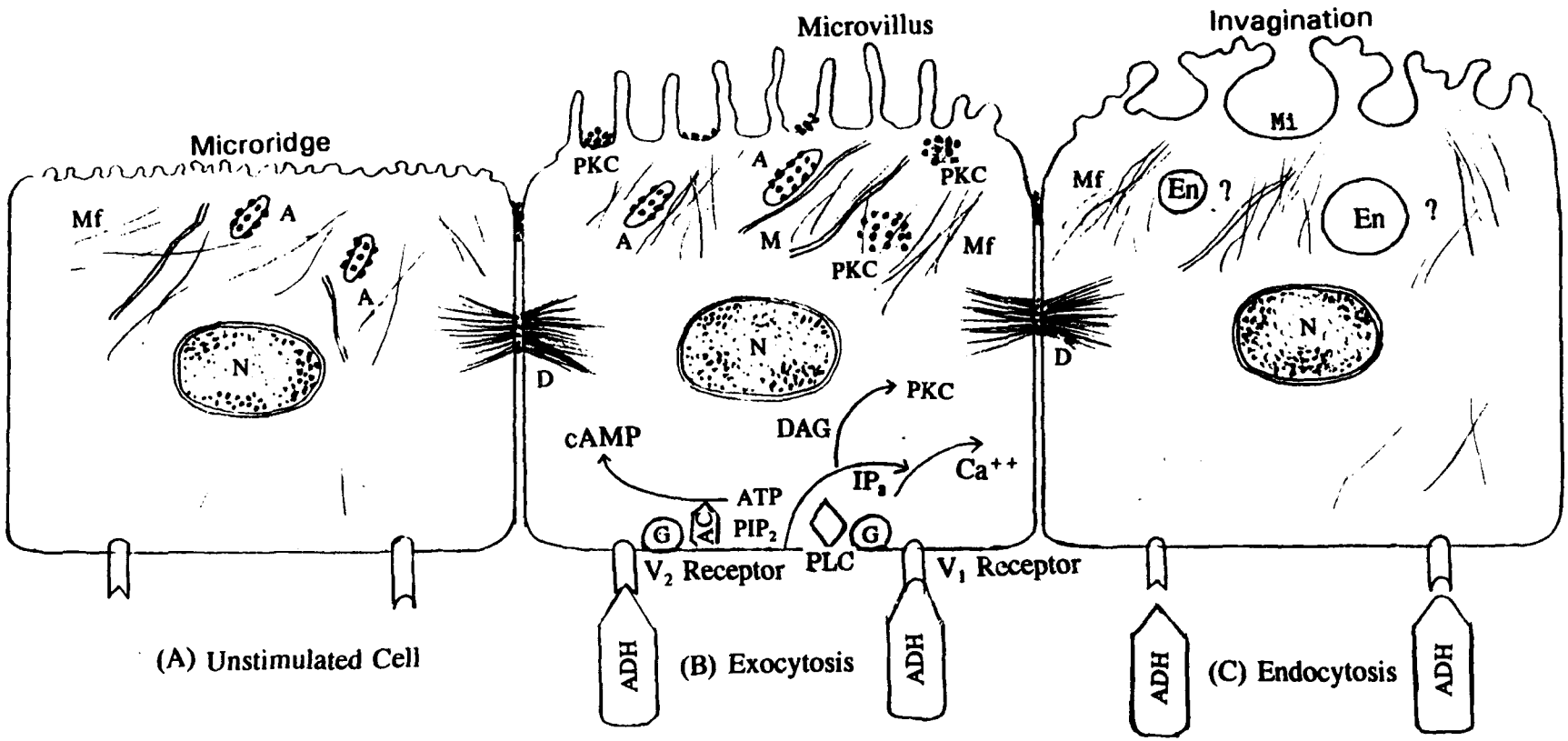


Fig. 1. Schematic representation of unstimulated (A), ADH or MZ stimulated (B) and retrieved cells (C) following withdrawal of ADH or MZ. A, aggrephores, D, desmosomes, En, endosomes, Mi, membrane invagination, M, microtubules, Mf, Microfilaments, N, nucleus, PKC, Protein Kinase C enzyme localized with protein A-gold particles.

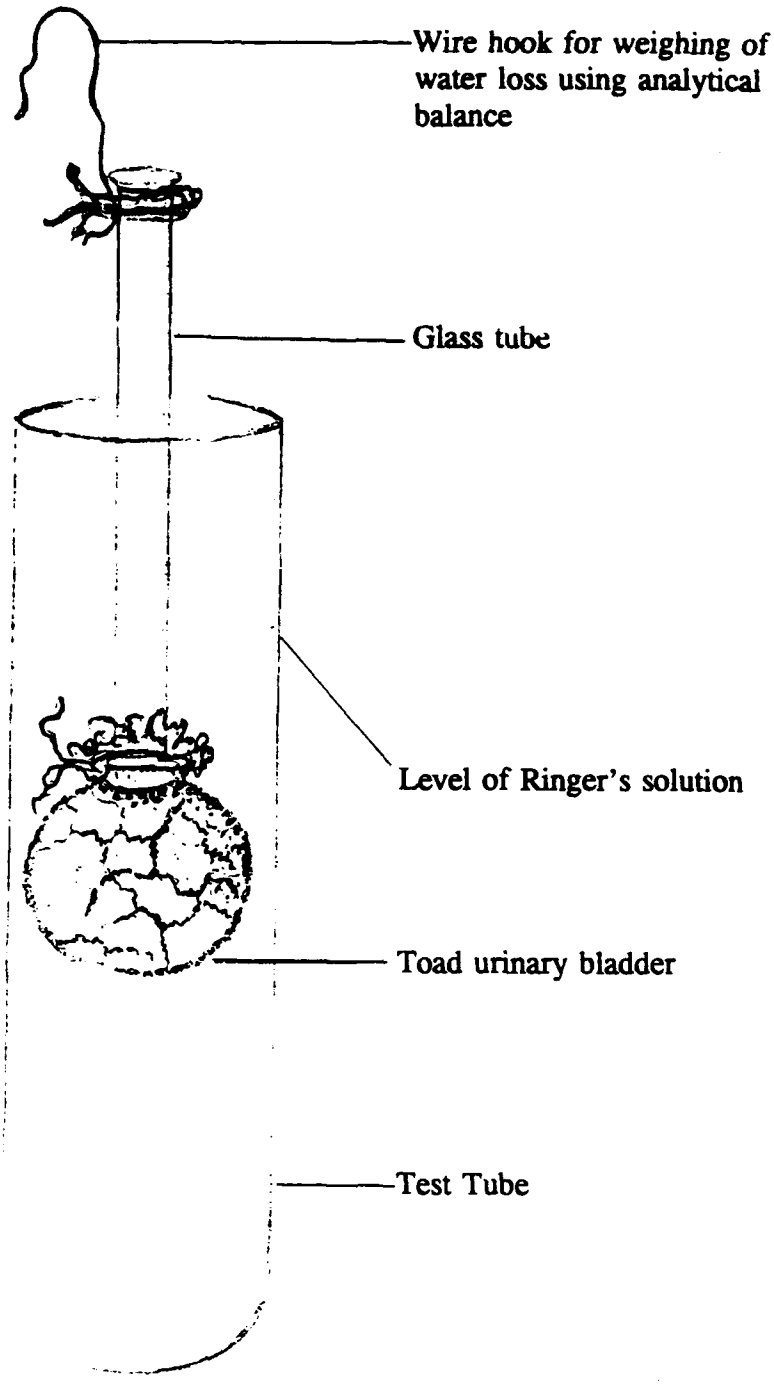
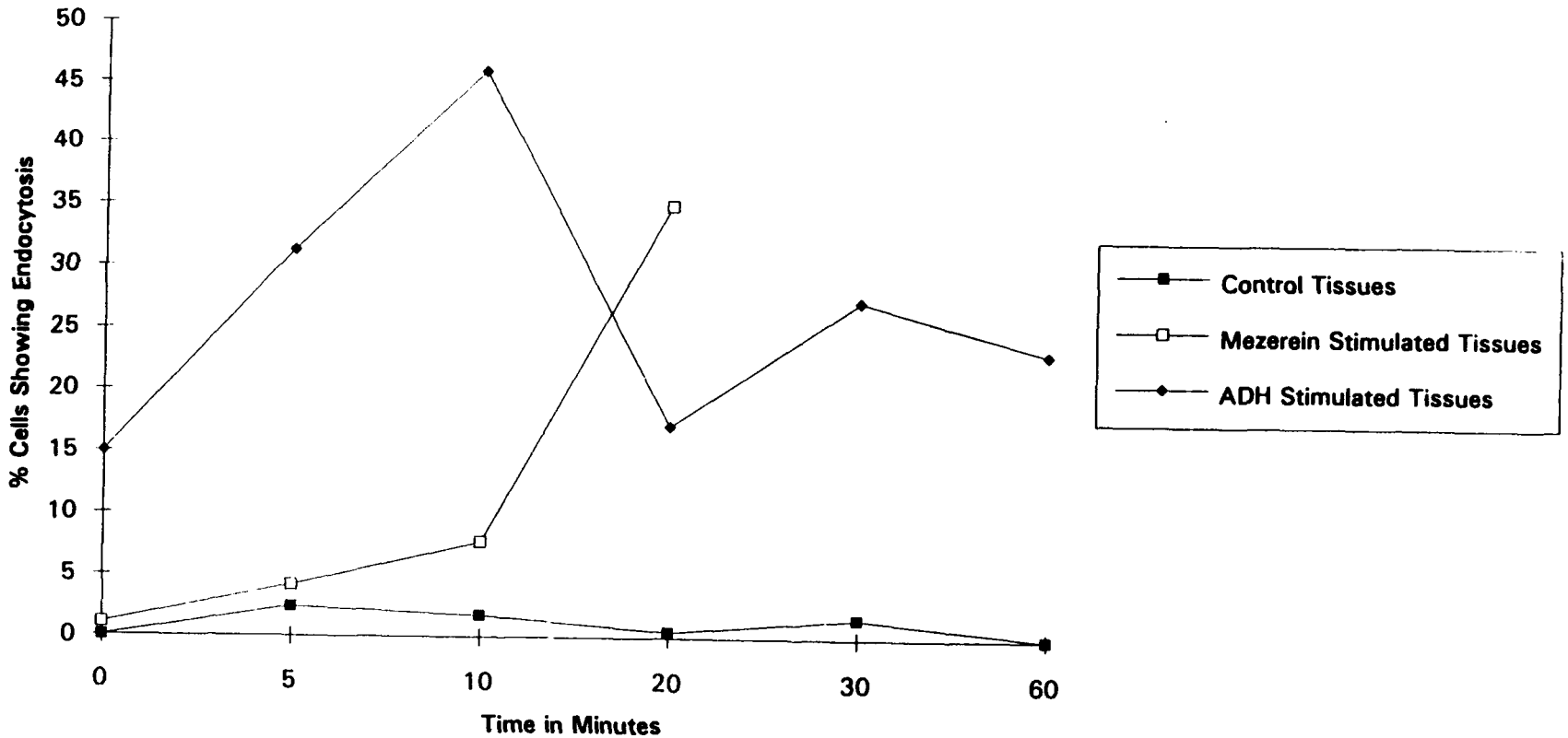
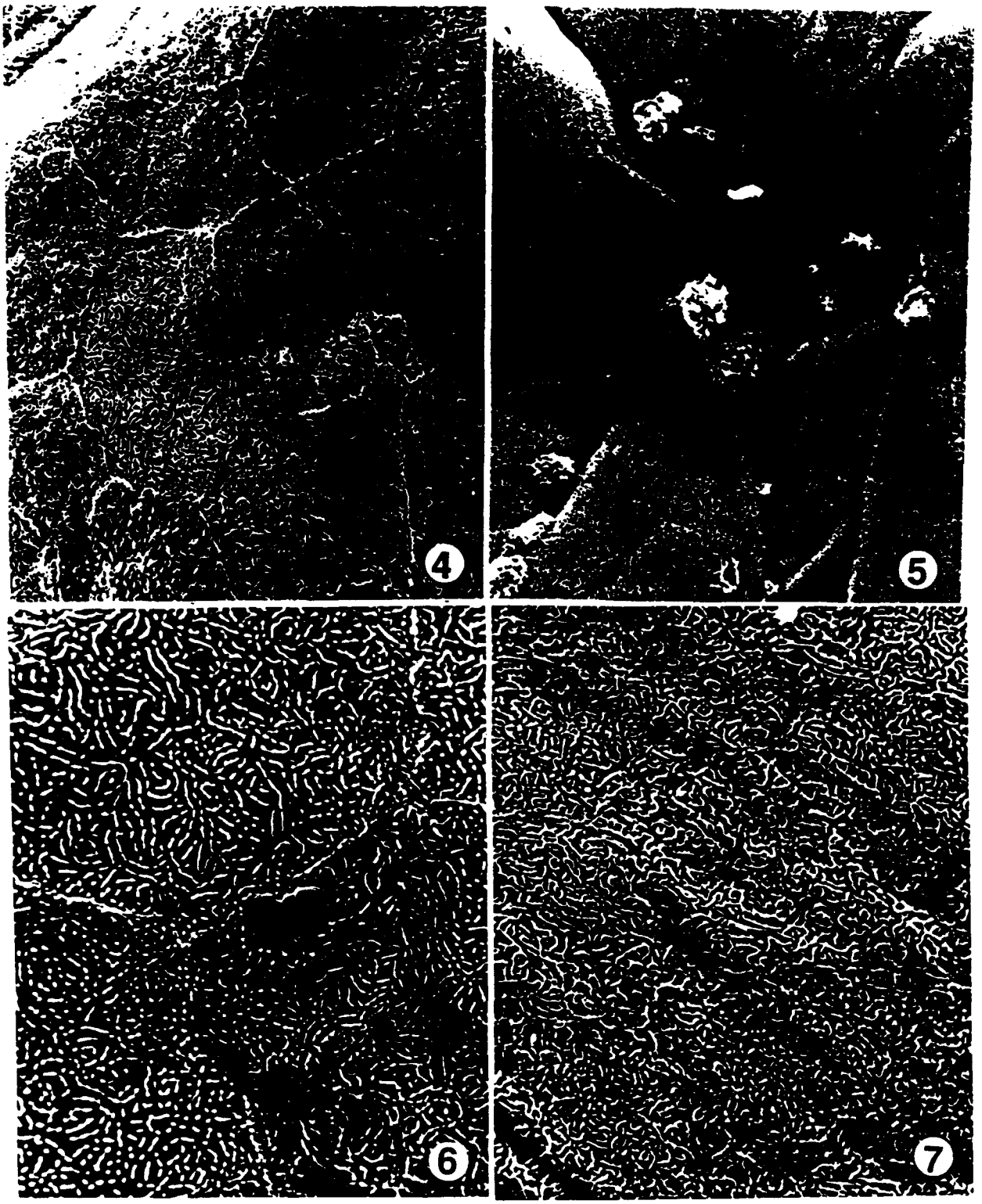


Fig. 2. Schematic representation of experimental set-up of toad urinary bladder as sac at the end of a glass tube.

Fig 3. Represents the Percentage of Endocytosed Cells Stimulated by ADH and MZ Compared to Control Tissues

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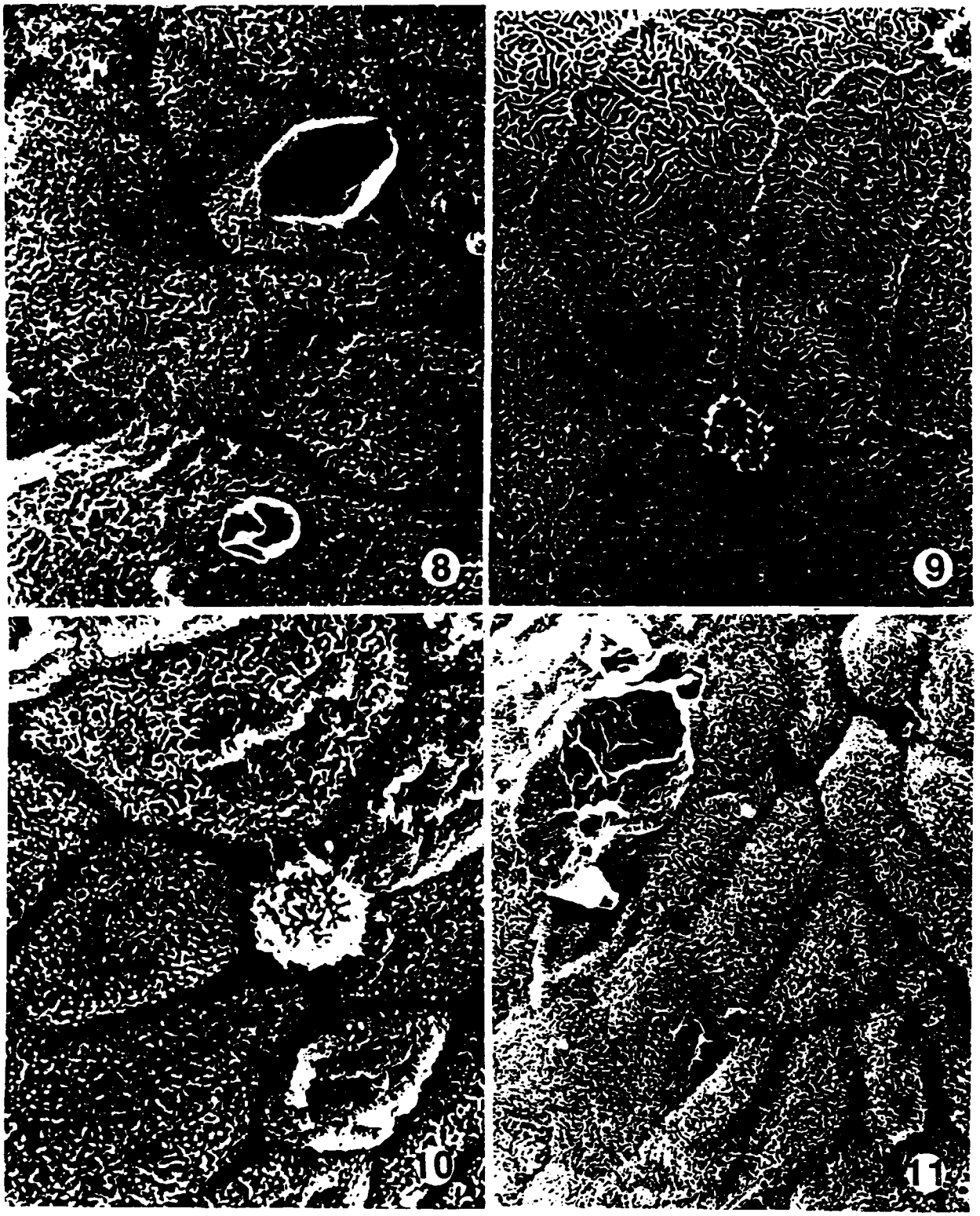
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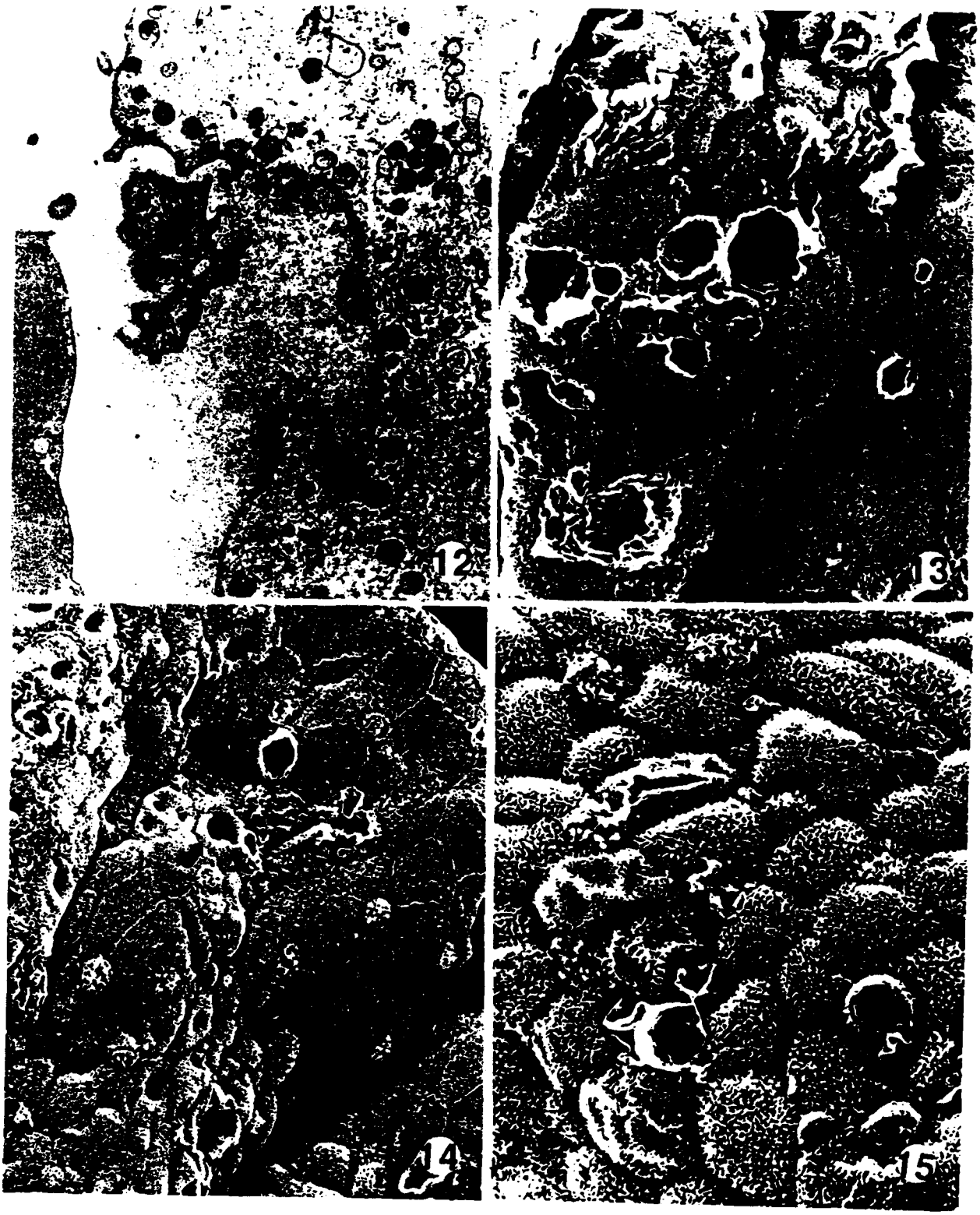
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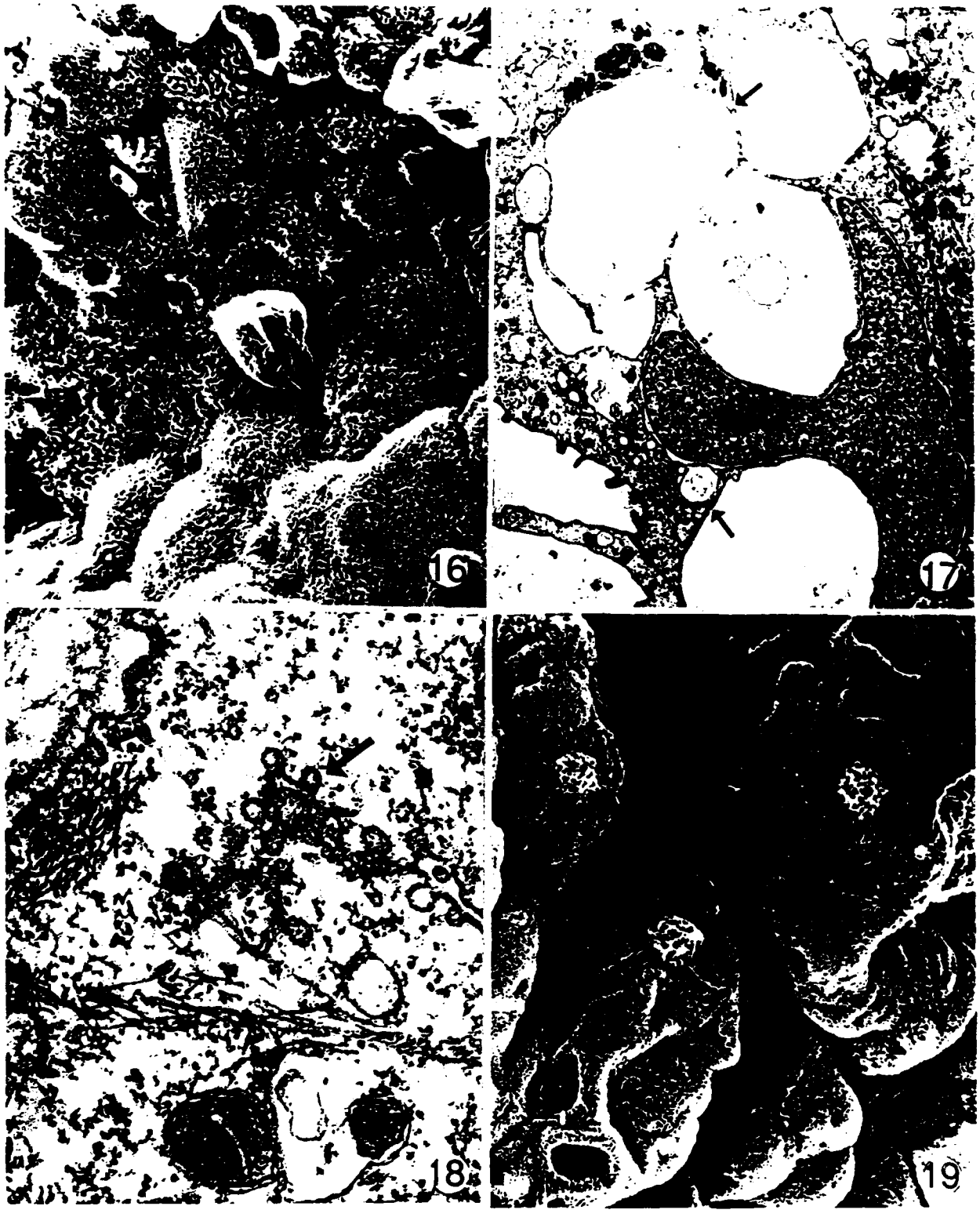
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